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Integrins induce expression of monocyte chemoattractant protein-1 via focal adhesion kinase in mesangial cells

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Integrins induce expression of monocyte chemoattractant protein-1 via focal adhesion kinase in mesangial cells.

Background. Integrins are major adhesion receptors that not only regulate cytoskeletal organization, but also trigger a variety of intracellular signal transduction pathways. We examined the effects of increased extracellular matrix (ECM) accumulation on monocyte chemoattractant protein-1 (MCP-1) expression, which is known to play an important role in the progression of various glomerular diseases.

Methods. MCP-1 mRNA and protein expression in cultured rat mesangial cells (MC) attached to ECM proteins were examined by reverse transcription (RT)-polymerase chain reaction (PCR) and Western blotting, respectively. Phosphorylation of focal adhesion kinase (FAK) was measured by Western blotting. Effects of wild-type and dominant-negative FAK on MCP-1 expression were examined by a transient transfection assay.

Results. Cell adhesion to fibronectin-induced phosphorylation of FAK and MCP-1 mRNA expression in time- and dose-dependent manners followed by increased MCP-1 protein expression. All integrin-interacting substrates (laminin and types I, III, and IV collagens) also increased levels of FAK phosphorylation and MCP-1 expression, whereas nonspecific adhesive substrates (polylysine and concanavalin A) had no significant effects. Overexpression of wild-type FAK increased phosphorylation of FAK and expression of MCP-1 mRNA and protein, whereas transfection of dominant-negative FAK abolished adhesion-induced MCP-1 expression. Adhesion-induced expression of MCP-1 mRNA was inhibited by genistein and tosyl phenylalanyl chloromethylketone (TPCK), suggesting that tyrosine kinases [e.g., FAK, and nuclear factor kappa B (NF-κB)] are necessary in this signaling.

Conclusion. Our results indicate that integrin-mediated cell adhesion to the ECM can induce MCP-1 expression through activation of FAK, and suggest a role for altered ECM deposition in the progression of glomerular diseases by affecting gene expression.

Key words: focal adhesion kinase, integrin, extracellular matrix, gene expression, glomerulonephritis.

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Glomerulosclerosis is the main pathologic feature of progressive glomerulonephritis (GN) and is characterized by abnormal accumulation of mesangial extracellular matrix (ECM). Abnormal remodeling of the ECM results from a complex process that involves increased expression of growth factors and cytokines, and leads to quantitative and constitutional changes in ECM components such as fibronectin, laminin, and collagen types I, III, and IV [1–4]. In addition, a correlation between glomerular expression of integrins (cell surface receptors for ECM proteins) and their ligand matrix components is also observed in various human and experimental GN [5–7], suggesting that altered cell-ECM interaction, consequent to enhanced expression of integrins and ECM proteins, may contribute to the progression of GN.

Integrins are heterodimeric receptors composed of pairs of α and β transmembrane subunits, and integrin-mediated adhesion to the extracellular matrix plays important roles in regulating formation of the cytoskeleton, cell survival, proliferation, and motility [8]. It has recently become clear that integrins are involved in both cell adhesion and signal transduction [9, 10]. Integrins can directly activate many intracellular signaling events after stimulation by ECM. Activation of intracellular signals includes tyrosine phosphorylation of focal adhesion kinase (FAK), which binds to the β1 integrin cytoplasmic domain and increases its tyrosine kinase activity upon binding to integrins [11]. Integrins and their cytoplasmic tails are involved in forming large complexes of cytoskeletal proteins and signaling molecules called focal adhesions that include FAK and other signal transduction molecules, such as p42/44 and p38 mitogen-activated protein (MAP) kinases, phosphatidylinositol 3-kinase (PI 3-K), protein kinase C (PKC), and c-Jun amino-terminal kinase (JNK) [12]. Furthermore, integrins can function in an additive or cooperative fashion with growth factors such as platelet-derived growth factor (PDGF) and angiotensin II [13, 14]. Thus, many of the well-known signaling pathways identified previously for growth factors and cyto-

kines are also activated by integrins and might lead to the induction of expression of specific genes.

Monocyte chemoattractant protein-1 (MCP-1) is a member of the chemokine family and has recently been reported to have a high degree of specificity as a chemotactic factor for monocytes/macrophages, and which plays an important role in the progression of inflammatory processes, including renal diseases [15]. In vitro studies have shown that MCP-1 is produced by kidney cells such as mesangial cells (MC) and tubular epithelial cells [16–18]. In fact, MCP-1 is implicated in the glomerular and tubulointerstitial injury of many renal diseases, including human idiopathic glomerular diseases [19] and hypertensive kidney [20], diabetic nephropathy [21, 22], and experimental renal diseases [23, 24]. Furthermore, inhibition of MCP-1 reduces monocyte/macrophage infiltration in antibody-induced experimental nephritis, indicating that MCP-1 might play an important role in the progression of renal diseases [25].

Recent studies have elucidated the mechanisms that regulate MCP-1 expression. Inflammatory cytokines and growth factors, such as interleukin-1 and PDGF, can trigger expression of MCP-1 through activation of several signaling pathways, such as p42/p44 and p38 MAP kinases [16, 26], PKC [27], activator protein-1 (AP-1) [28], and nuclear factor kappa B (NF-κB) [29]. In addition, our recent study showed that high pressure loading of MC induced MCP-1 expression [16], suggesting that mechanical strain such as glomerular hypertension itself can up-regulate MCP-1 expression. Recently, it was reported that cell adhesion to ECM proteins induces MCP-1 expression in hepatic stellate cells [30] and endometrial stromal cells [31]. However, it remains unknown whether integrin-mediated cell adhesion can stimulate MCP-1 expression in MC, and the molecular mechanism by which cell adhesion induces MCP-1 expression is not yet elucidated. In the present study, we provide evidence that integrin-mediated cell adhesion to ECM proteins induces MCP-1 expression through activation of FAK in MC. These data invoke a novel mechanism for regulation of MCP-1 in MC and support the possible role for abnormal ECM accumulation in the pathophysiology of glomerular diseases through the induction of expression of specific genes.

METHODS

Materials

Human fibronectin, laminin, type I, III, and IV collagens, poly-L-lysine, and concanavalin A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Petri dishes were coated by incubating with 10 μg/mL or the indicated amount of ECM proteins or adhesive substrates in phosphate-buffered saline (PBS) for 1 hour at 37°C, washed three times with PBS, and used for cell adhesion assays. Monoclonal antibodies for FAK and hemagglutinin (HA) were obtained from Transduction Laboratories (Lexington, KY, USA) and Boehringer Mannheim (12CA5; Indianapolis, IN, USA), respectively. Polyclonal antibodies against Y397-phosphorylated FAK and rat MCP-1 were from BioSource International (Camarillo, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Tyrosine kinase inhibitor genistein, MEK1 inhibitor, PD98059, and PKC inhibitor, calphostin C, were purchased from Calbiochem (San Diego, CA, USA). To inhibit NF-κB activation, protease inhibitor tosyl phenylalanyl chloromethylketone (TPCK) (Sigma Chemical Co.) or antioxidant pyrrolidinedithiocarbamate (PDTC) (Sigma Chemical Co.) were used.

Cell culture

Glomerular MC from male Wistar rats were isolated by the differential sieving method and identified by their positive immunostaining for vimentin, smooth muscle–specific actin, and negative staining for cytokeratins, factor VIII–related antigen, and leukocyte common antigen (antibodies from Dako Corp., Carpinteria, CA, USA), as described [32]. MC were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin, and amphotericin B at 37°C in a 5% CO_2 incubator. To synchronize the cells in quiescence, MC were maintained in DMEM containing 0.5% FCS for 24 hours prior to the experiments. MC were used between passages 5 and 15.

Reverse transcription-polymerase chain reaction (RT-PCR)

mRNA was extracted with the QuickPrep mRNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The first-strand cDNA was synthesized from 25 ng of poly(A)$^+$ RNA in 50 mmol Tris-HCl buffer (pH 8.3) containing 200 ng random hexamers, 3 mmol $MgCl_2$, 400 U murine Moloney leukemia virus reverse-transcriptase, 500 μmol desoxynucleoside triphosphate (dNTP), 15 mmol dithiothreitol (DTT), and 75 mmol KCl in a final volume of 15 μL for 1 hour at 37°C using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). Each sample was assayed for MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA using specific primers for polymerase chain reaction (PCR) as described [16]: MCP-1; sense; 5′-TATGCAG GTCTCTGTCACGC-3′, antisense; 5′-AAGTGTTGA ACCAGGATTCACA-3′, GAPDH; sense; 5′-TCCCT CAAGATTGTCAGCAA-3′, antisense; 5′-AGATCC ACAACGGATACATT-3′. PCR was performed by incubating 25 ng of the sample cDNA with 50 mmol KCl, 10 mmol Tris-HCl (pH 8.3), 1.5 mmol $MgCl_2$, 0.01% gelatin, 2.5 U Taq DNA polymerase, 400 μmol dNTP, and 40 pmol of primers in a final volume of 50 μL. PCR was performed for 26 cycles for MCP-1 and 22 cycles for GAPDH under the following conditions: 1 minute

at 95°C, 45 seconds at 60°C, and 45 seconds at 72°C. Primer sets for MCP-1 and GAPDH generated 595 bp and 308 bp products, respectively. The samples were subjected to agarose gel electrophoresis and stained with ethidium bromide to visualize DNA bands, followed by scanning densitometry (FB1200S; Canon, Tokyo, Japan).

Cell spreading assay

Cell spreading on extracellular matrix was quantified as described previously [33]. Briefly, MC were allowed to spread on Petri dishes coated with 10 μg/mL fibronectin for 3 hours and then fixed with 4% paraformaldehyde in PBS. The percentage of cells spread on matrix was determined by scoring at least 100 cells per sample in a double-blinded fashion.

Western blotting

Cells plated on 10 μg/mL fibronectin-coated dishes were solubilized in radioimmunoprecipitation assay (RIPA) buffer [50 mmol Tris, pH 7.4, 150 mmol NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mmol ethyleneglycol tetraacetate (EGTA), 1 mmol phenylmethylsulfonyl fluoride, 1 mmol sodium orthovanadate, 1 mmol sodium fluoride, and a protease inhibitor mixture (Complete™; Boehringer Mannheim)]. Homogenates were clarified by centrifugation at 15,000*g* for 15 minutes at 4°C, and equal amounts of total cell lysates (30 μg protein) were analyzed by Western blotting using anti-phospho FAK (1:2000), total FAK (1:1000), or MCP-1 (1:2000) antibodies. Blots were visualized by the enhanced chemiluminescence (ECL) reaction (Amersham Life Science). Phosphorylation of FAK was determined as a ratio of phosphorylated FAK against total FAK levels.

Plasmids and transfection assay

Plasmid-containing HA-tagged FAK (wild-type) was kindly provided by Dr. J. Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health) [33]. The point mutant Y397F was introduced into FAK by site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene, CA, USA) [34] and kindly provided by Dr. Kenneth M. Yamada (National Institute of Dental and Craniofacial Research, National Institutes of Health). Puromycin-resistant plasmid pHA262pur was obtained from Dr. Heinte Riele (Division of Molecular Carcinogenesis, The Netherlands Cancer Institute, Amsterdam, The Netherlands). Either wild-type or dominant-negative FAK (8 μg) was transfected into MC using the cationic liposome- (LipoTAXI® Mammalian Transfection Kit; Stratagene) mediated transfection method together with pHA262pur (1 μg) [16, 32]. To increase the expression of transfected genes, 5 mmol sodium butyrate was added to the culture medium for 24 hours after transfection [34, 35]. Cells expressing HA-FAK were selected as described [16]. Briefly, at 24 hours after transfection, cells were maintained for two days in culture medium containing 1 μg/mL puromycin, and cultured for an additional 24 hours in the regular culture medium containing 0.5% serum without puromycin. Equal expression of each HA-FAK plasmid was confirmed by Western blotting using anti-HA antibody.

Statistical analysis

Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using analysis of variance (ANOVA). A P value of less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Cell adhesion–induced MCP-1 mRNA expression

To confirm the accuracy of the mRNA quantity amplified by RT-PCR, PCR for MCP-1 and GAPDH was performed by differential PCR cycles or by incubating serially increasing amounts of mesangial cDNA synthesized from 25 ng of mRNA (data not shown), which revealed a dose- and cycle-dependent increase in the PCR product. We therefore amplified 25 ng of each mRNA for 26 cycles for MCP-1 or 22 cycles for GAPDH in the following experiments.

First, we examined the dynamics of the effects of cell adhesion to the matrix on the expression of MCP-1 mRNA expression (Fig. 1). MCP-1 mRNA was expressed at low levels in serum-starved and nonadherent MC; however, it rapidly increased from 30 minutes with a peak noted at 3 to 6 hours. We also examined the effect of different amounts of fibronectin on MCP-1 mRNA expression. Although few cells were spread on fibronectin at 1 or 2.5 μg/mL, cell spreading on fibronectin was significantly increased when cells were spread on more than 5 μg/mL fibronectin (Fig. 2A). The increase in cell spreading was similar to the increase in MCP-1 mRNA expression at the same fibronectin concentration (Fig. 2B). Although there were no significant increases in MCP-1 mRNA expression in cells spread on less than 2.5 μg/mL fibronectin, MCP-1 mRNA expression increased significantly when the cells were spread on more than 5 μg/mL fibronectin.

In addition, we examined MCP-1 protein expression after adhesion to 10 μg/mL fibronectin for the indicated times (Fig. 3 A and B). MCP-1 protein levels in MC increased significantly at 3 hours after adhesion, and reached a peak level at 12 hours following MCP-1 mRNA expression, reaching a peak level at 3 to 6 hours (Fig. 1). In contrast, when cells were plated on polylysine, which mediates integrin-independent cell adhesion, there was no significant increase in MCP-1 protein expression (Fig. 3B).

Integrin-mediated expression of MCP-1 mRNA

We next examined whether cell adhesion–mediated expression of MCP-1 was specific to integrin-mediated

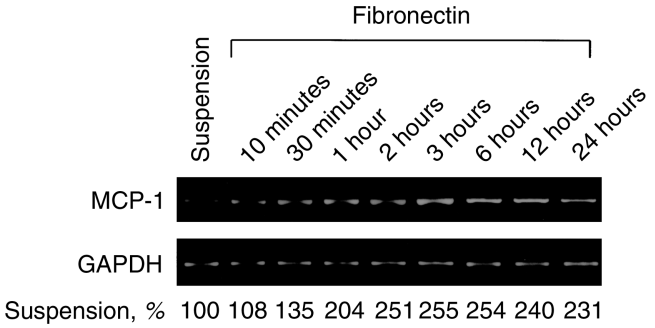

Fig. 1. Expression of MCP-1 mRNA by fibronectin. Cultured MC were serum-starved for 24 hours and detached from the dishes by trypsin/EDTA digestion. Cells were then plated on 10 μg/mL fibronectin-coated dishes for the indicated times in 5% bovine serum albumin containing DMEM without serum. mRNAs (25 ng) obtained from cells in suspension or cells plated on fibronectin (10 μg/mL) for up to 24 hours were assayed for the expression of MCP-1 and GAPDH mRNA. The amount of the PCR product was determined by scanning densitometry and the relative ratio of MCP-1 to GAPDH band density was calculated for each lane. Values represent mean MCP-1 mRNA expression levels expressed as the relative ratio of MCP-1 to GAPDH from five independent experiments. Data are expressed as a percentage of the amount of cells in suspension. Abbreviations are: MCP-1, monocyte chemoattractant protein-1; MC, mesangial cells; EDTA, ethylenediaminetetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; GADPH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.

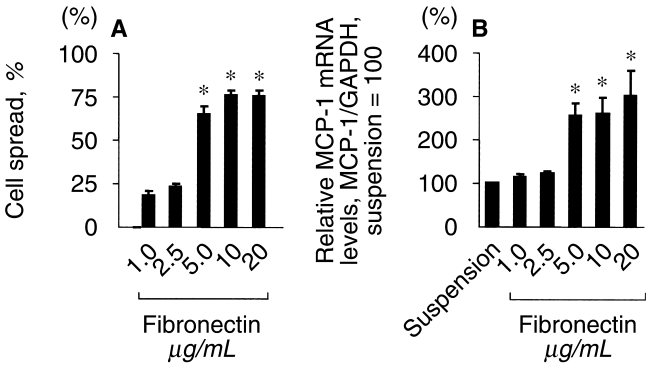

Fig. 2. Dose-dependence of fibronectin-induced MCP-1 mRNA expression. (*A*) Dose-dependent cell adhesion to fibronectin. Cultured MC were allowed to spread on petri dishes coated with the indicated concentration of fibronectin for 3 hours. The percentage of cells spread on fibronectin was determined as described in **Methods**. Data represent the mean ± SD of four independent experiments. (*B*) Dose-dependence of fibronectin-induced MCP-1 mRNA expression. After cell spreading on the indicated concentration of fibronectin for 3 hours, expression of MCP-1 mRNA was analyzed by RT-PCR (suspension = 100). Data represent the mean ± SD of four independent experiments. $^*P < 0.01$ vs. suspension. Abbreviations are: MCP-1, monocyte chemoattractant protein-1; MC, mesangial cells; RT-PCR, reverse transcription polymerase chain reaction.

cell adhesion. Cell interactions with ECM proteins through integrin receptors can mediate transmembrane signal transduction, including integrin-mediated tyrosine phosphorylation of FAK [9]. Phosphorylation of FAK was determined as the ratio of phosphorylated FAK against total FAK levels by Western blotting using anti-phospho Y397 FAK and total FAK antibodies. Cell adhesion to

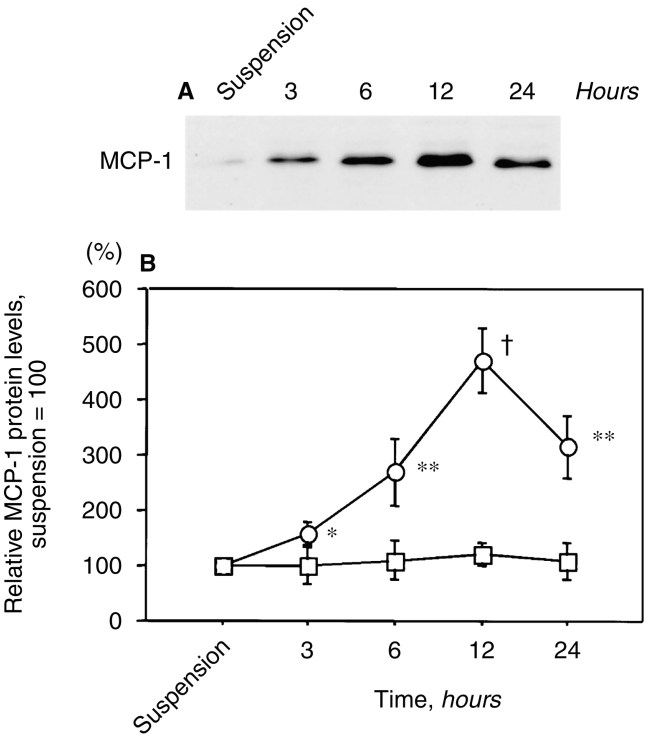

Fig. 3. Expression of MCP-1 protein by cell adhesion to fibronectin. (*A*) MCP-1 protein expression in cells in suspension or in cells plated on fibronectin (10 μg/mL) for the indicated times was analyzed by Western blotting. (*B*) The amount of MCP-1 protein was determined by scanning densitometry and expressed as a ratio against the level of cells in suspension. Values represent mean ± SD of four independent experiments. Symbols are: (○), cells plated on 10 μg/mL fibronectin; (□), cells plated on 10 μg/mL polylysine. $^*P < 0.05$; $^{**}P < 0.01$; †$P < 0.005$ vs. suspension. MCP-1 is monocyte chemoattractant protein-1.

fibronectin rapidly increased FAK phosphorylation and reached a peak level at 1 hour (Fig. 4). All integrin-interacting matrix components, such as fibronectin, laminin, and type I, III, and IV collagens significantly increased FAK phosphorylation and MCP-1 mRNA expression (Fig. 5 A and B). However, the nonspecific adhesive substrates, polylysine and concanavalin A, had no significant effect on either MCP-1 mRNA expression or FAK phosphorylation, suggesting that cell adhesion–induced expression of MCP-1 depends on specific integrin-mediated cell adhesion to ECM. Interestingly, although the physiologic collagen, type IV, significantly increased both MCP-1 mRNA expression and FAK phosphorylation, compared to those of cells in suspension, the levels were less than those of the pathologic collagens, types I and III (Fig. 5 A and B).

Role of FAK on integrin-mediated expression of MCP-1 mRNA

FAK is one of the key molecules that transduces integrin-mediated signals from the ECM. Cell adhesion–induced FAK phosphorylation followed by MCP-1 ex-

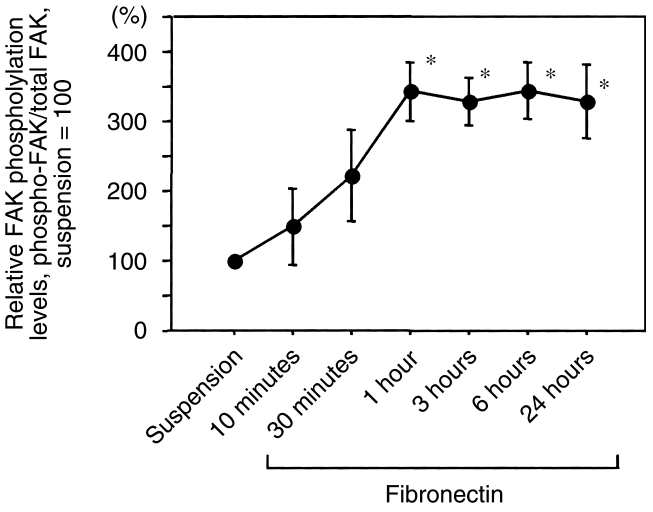

Fig. 4. Effects of cell adhesion to fibronectin on FAK phosphorylation. MCP-1 protein expression in cells in suspension or cells plated on fibronectin (10 μg/mL) for the indicated times was analyzed by Western blotting using anti-phospho Y397 FAK antibody. Relative FAK phosphorylation levels were determined from Western blotting by densitometric analysis and expressed as the relative ratio of phospho-FAK to total FAK. Values represent mean ± SD of four independent experiments. $*P < 0.01$ vs. suspension. FAK is focal adhesion kinase.

pression suggests that the expression of MCP-1 may be a downstream event of FAK phosphorylation. Next, we tested whether overexpression of wild-type FAK could enhance cell adhesion–induced expression of MCP-1 and whether expression of a dominant-negative form of FAK could, in turn, attenuate cell adhesion–induced MCP-1 expression. We cotransfected HA-FAK or its mutant with a plasmid containing the puromycin resistance gene and selected transfectants for 2 days using puromycin as described previously [16]. This puromycin selection procedure routinely yielded ~90% pure populations of transfectants, according to fluorescence analyses using HA markers. After selection by puromycin, the selected cells were further analyzed for HA-tagged FAK constructs by Western blotting. As shown in Figure 6A (lower panel), expression of wild-type FAK and dominant-negative FAK (Y397F) were recognized by anti-HA antibody. In addition, overexpression of FAK constructs resulted in increases in total FAK protein levels by Western blotting using anti-total FAK antibody that recognizes both endogenous and HA-FAK (Fig. 6A, middle panel) as described previously [36]. Although FAK phosphorylation was increased in cells transfected with no FAK construct when cells were plated on fibronectin, cells transfected with Y397F FAK exhibited marked suppression of FAK phosphorylation (Fig. 6A, upper panel, and Fig. 6B). Furthermore, overexpression of wild-type FAK resulted in increased FAK phosphorylation. Levels of FAK phosphorylation were increased to 348% and 476% in cells transfected without or with wild-type FAK, respectively, while cells transfected with Y397F FAK showed no significant increase in FAK phosphorylation (Fig. 6B). Furthermore, enhancement or suppression of MCP-1 mRNA and protein were similarly observed in cells transfected with wild-type or Y397F FAK (Fig. 7 A to C), indicating that expression of MCP-1 is a downstream event of FAK activation.

Effects of various inhibitors on MCP-1 mRNA expression

FAK has been reported to activate downstream signaling molecules such as MAP kinase, PKC, PI-3 kinase, and NF-κB [9, 37]. We further examined the downstream signaling pathways below FAK, because it has been reported that inflammatory cytokines, growth factors, and mechanical forces can induce expression of MCP-1 through activation of several signaling pathways, such as PKC [27], NF-κB [29], and MAP kinase [16]. To inhibit NF-κB activation, MC were pretreated with the protease inhibitor TPCK, which has been shown to inhibit interleukin-1–inducible NF-κB in MC and other cells [29]. Antioxidant pyrrolidine dithiocarbamate (PDTC) has been shown to block phorbol ester–induced NF-κB activation in several cell lines [29] and was also tested. Adhesion-induced expression of MCP-1 mRNA was suppressed totally by genistein, a tyrosine kinase inhibitor, and TPCK, partially by PD98059, a MEK1 inhibitor, and calphostin C, a PKC inhibitor, but not by wortmannin, a PI3-kinase inhibitor (Fig. 8A). These results suggest that tyrosine kinases such as FAK and NF-κB are necessary for integrin-mediated induction of MCP-1, and that MEK1 and PKC are partially involved in this signaling. Interestingly, PDTC did not attenuate MCP-1 induction, similar to the observation that PDTC did not block IL-1–induced NF-κB activation, whereas TPCK completely inhibited such activation [29]. Furthermore, TPCK abolished wild-type FAK-induced enhancement of MCP-1 expression, suggesting that NF-κB is located downstream of FAK in integrin-mediated MCP-1 expression (Fig. 8B).

DISCUSSION

Integrins mediate a variety of biologic processes, such as cell adhesion, migration, and invasion, by functioning as receptors for ECM molecules. In addition, integrin interaction with ECM ligands also triggers transmembrane effects on the localization of signaling molecules, as well as cytoskeletal molecules into focal adhesions [12]. Thus, it activates signaling pathways and eventually leads to regulation of gene expression, such as immediate early genes and inflammatory cytokines [38, 39]. To our knowledge, this study demonstrates for the first time that integrin-mediated cell adhesion to ECM can induce MCP-1 expression in MC, and that these effects of integrins on MCP-1 expression depend on the activation of

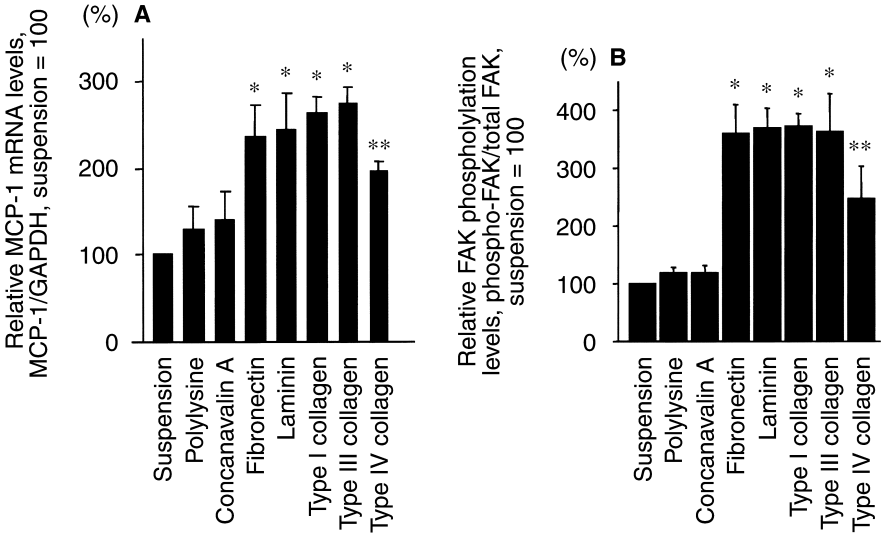

Fig. 5. Effects of various extracellular matrices on MCP-1 mRNA expression and FAK phosphorylation. (*A*) Effects of various extracellular matrices on MCP-1 mRNA expression. Cultured MC spread on indicated matrix proteins (10 μg/mL each) for 3 hours were analyzed for MCP-1 mRNA expression by RT-PCR. Data represent the mean ± SD of four independent experiments. $*P < 0.01$; $**P < 0.05$ vs. suspension. (*B*) Effects of various extracellular matrices on FAK phosphorylation. Cultured MC spread on indicated matrix proteins (10 μg/mL each) for 3 hours were analyzed for FAK phosphorylation by Western blotting using anti-phospho Y397 FAK antibody. Data represent the mean ± SD of four independent experiments. $*P < 0.01$; $**P < 0.05$ vs. suspension. Abbreviations are: MCP-1, monocyte chemoattractant protein-1; FAK, focal adhesion kinase; MC, mesangial cells; RT-PCR, reverse transcription polymerase chain reaction.

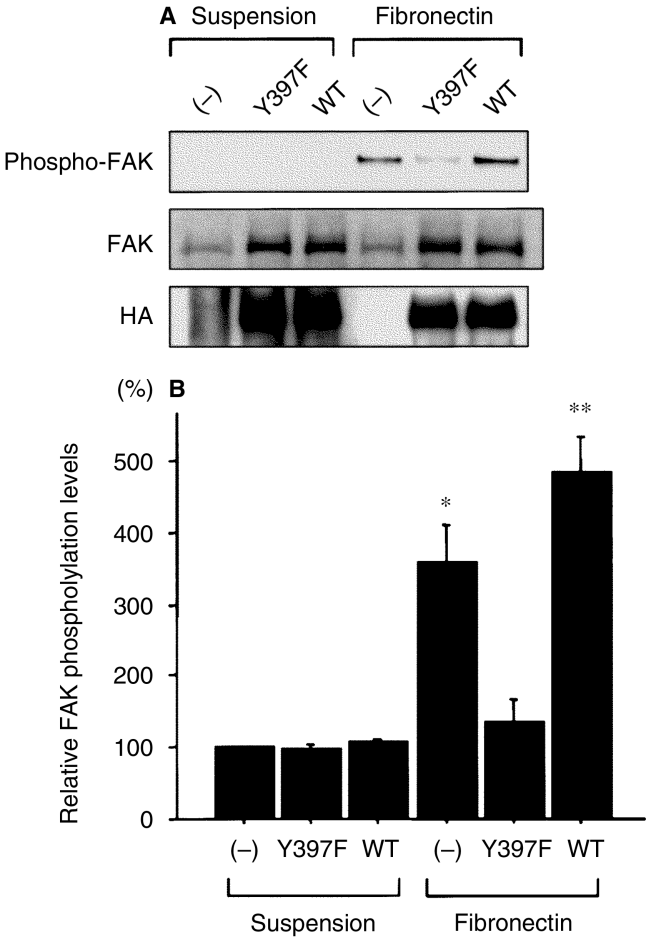

Fig. 6. Effects of wild-type and dominant-negative FAK on FAK phosphorylation. (*A*) MC were transfected without or with either HA-FAK (Y397F) or HA-FAK (wild-type) plasmid together with puromycin-resistant gene and transfectants were selected by puromycin as described in **Methods**. Levels of FAK phosphorylation and amounts of total FAK and HA expression were analyzed by Western blotting in cells in suspension or in cells spread on fibronectin (10 μg/mL) for 3 hours. (*B*) Relative FAK phosphorylation levels were determined from Western blotting by densitometric analysis and expressed as the ratio against levels of cells in suspension without FAK constructs. Values represent mean ± SD of four independent experiments. $*P < 0.01$ vs. suspension. $**P < 0.05$ vs. cells without FAK constructs plated on fibronectin. Abbreviations are: FAK, focal adhesion kinase; MC, mesangial cells; HA, hemagglutinin.

FAK. These results suggest the importance of abnormal ECM accumulation in modulating the pattern of synthesis of a chemokine by glomerular MC.

FAK is a key molecule implicated in integrin signaling pathways. The activation of integrins by cell binding to ECM leads to the association of β1 integrin cytoplasmic domain and N-terminal domain of FAK, followed by an increase in FAK tyrosine phosphorylation and enhancement of its kinase activity [9, 40, 41]. It has been suggested that activation of FAK is required for the induction of integrin-mediated MCP-1 activation in hepatic stellate cells and endometrial stromal cells, because treatment of cells with cytochalasin D, which disrupts the actin cytoskeleton and blocks tyrosine phosphorylation of FAK, inhibits the cell adhesion–dependent increase in MCP-1 expression [30, 31]. In the present study, tyrosine phosphorylation of Y397 in FAK, which is a key tyrosine phosphorylation site for full activation of FAK [40], was observed prior to MCP-1 mRNA expression. Furthermore, overexpression of wild-type FAK enhanced the matrix-induced MCP-1 expression, while dominant-negative FAK completely inhibited MCP-1 expression. Thus, manipulating the amount of phosphorylated FAK could result in changes in MCP-1 expression. These results strongly

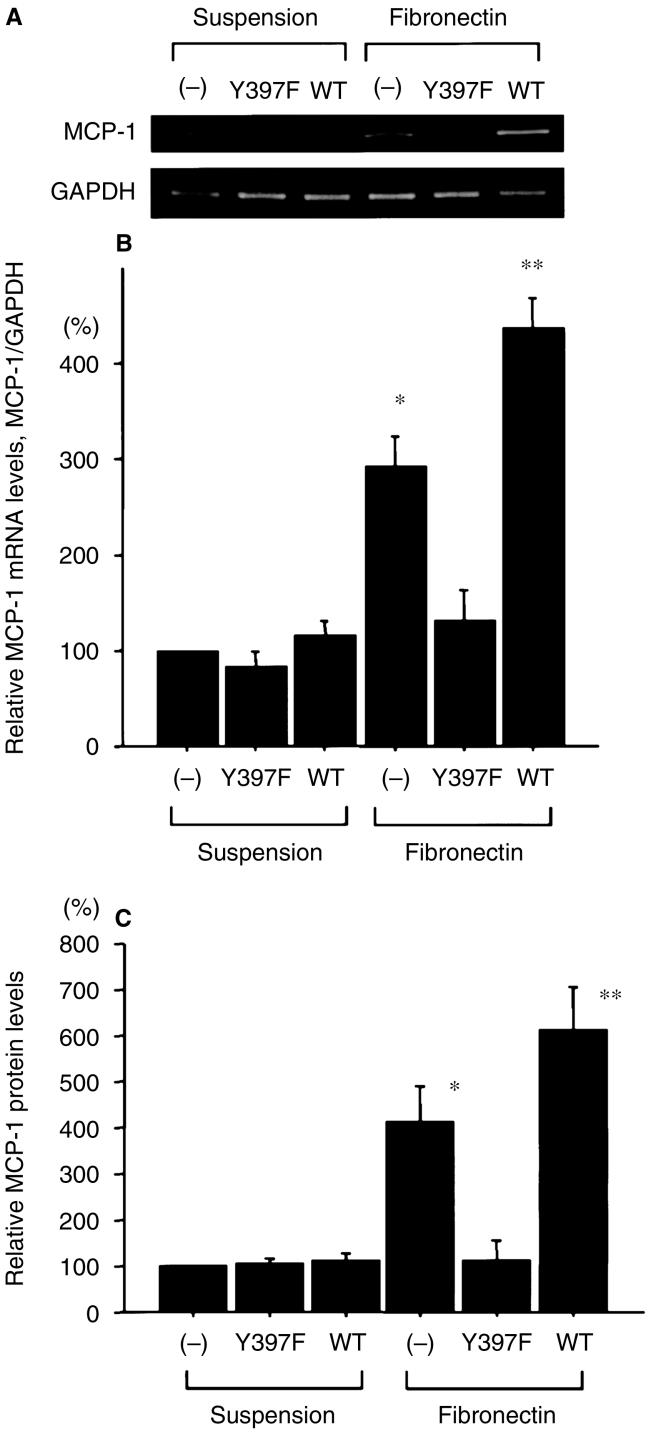

Fig. 7. Effects of wild-type and dominant-negative FAK on MCP-1 mRNA and protein expression. (*A*) MC were transfected without or with either HA-FAK (Y397F) or HA-FAK (wild-type) plasmid together with puromycin-resistant gene as described in Figure 7. Each transfectant, either in suspension or plated on fibronectin (10 μg/mL) for 3 hours, was assayed for MCP-1 and GAPDH mRNA expression by RT-PCR. (*B*) Relative MCP-1 mRNA expression levels were determined by densitometric analysis and expressed as the ratio against levels of cells in suspension. Values represent mean ± SD of four independent experiments. (*C*) Each transfectant, either in suspension or plated on fibronectin (10 μg/mL) for 12 hours, was also assayed for MCP-1 protein expression by Western blotting. The amount of MCP-1 protein was determined by scanning densitometry and expressed as a ratio against the level of cells in suspension without HA-FAK plasmids. Values represent mean ± SD of three independent experiments. $^{*}P < 0.01$ vs. suspension; $^{**}P < 0.05$ vs. cells without FAK constructs plated on fibronectin. Abbreviations are: FAK, focal adhesion kinase; MCP-1, monocyte chemoattractant protein-1; MC, mesangial cells; HA, hemagglutinin; GADPH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction.

indicate that integrin-mediated cell adhesion induces MCP-1 expression in MC through activation of FAK.

In the present study, FAK phosphorylation was observed within 10 minutes of cell attachment to the matrix, reaching a peak level at 1 hour, while expression of MCP-1 mRNA and protein appeared after 30 minutes and 3 hours, respectively, reaching peak levels at 3 to 6 and 12 hours, respectively. Thus, cell adhesion to the matrix triggered a sequential induction of FAK phosphorylation and expression of MCP-1 mRNA and protein. The time courses of MCP-1 mRNA and protein expression observed in this study were similar to those of previous studies in hepatic stellate cells, in which MCP-1 mRNA appeared after 1 hour and peaked at 4 hours [30], and in endometrial stromal cells, in which MCP-1 protein was expressed from 12 to 24 hours [31].

Cells plated on all integrin-interacting substrates examined in this study (fibronectin, laminin, and type I, III, and IV collagens) showed substantial induction of FAK phosphorylation and MCP-1 expression, whereas the nonspecific adhesive substrates, polylysine and concanavalin A, had no significant effects, indicating that cell adhesion–induced expression of MCP-1 depends on integrin-mediated cell adhesion to the ECM. Interestingly, both FAK phosphorylation and MCP-1 expression strongly increased in cells seeded on fibronectin, laminin, and collagen types I and III when compared with cells on collagen type IV. These results were similar to those in endometrial stromal cells, in which fibronectin and laminin induced increases of 6- and 4-fold in MCP-1 mRNA, respectively, whereas collagen type IV induced an increase of only 2-fold [31]. Similar differences between collagen types I and IV were also reported by Gonzalez-Santiago et al [42]; collagen type I showed an increase in endothelin 1 expression when compared with collagen type IV in endothelial cells. Although further study is necessary to elucidate the mechanisms underlying these differences, differential activation of integrin subunits could be responsible for the observed differences between the ECM proteins tested in this study. The integrin family is composed of pairs of α and β subunits, which are selected from among at least 16 α and 8 β subunits to form more than 20 different αβ heterodimeric receptors on cell surfaces [10, 43]. Thus, each subunit contributes to ligand specificity of the integrins. Both α1β1 and α2β1 integrins are collagen recep-

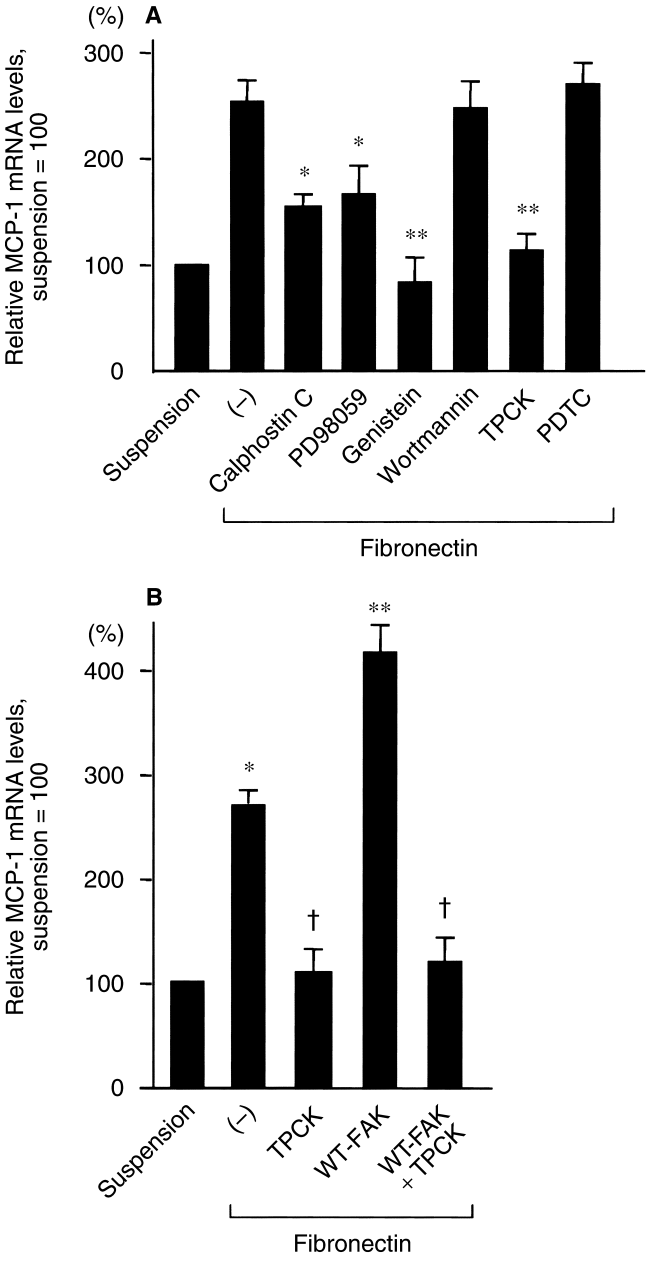

Fig. 8. Effects of various inhibitors on MCP-1 mRNA expression. (*A*) Cultured MC were pretreated with or without the indicated inhibitors (calphostin C, 1 μmol; PD98059, 25 μmol; genistein, 5 μmol; wortmannin, 500 nmol; TPCK, 25 μmol; PDTC, 50 μmol) for 30 minutes before and during spreading on fibronectin (10 μg/mL) for 3 hours. Expression of MCP-1 mRNA was analyzed by RT-PCR. Relative MCP-1 mRNA expression levels were determined as described in Figure 7. Data represent the mean ± SD. *$P < 0.05$ vs. suspension or fibronectin without inhibitors; **not significant vs. suspension. (*B*) Cells transfected with or without HA-FAK (wild-type) plasmid were maintained in suspension or plated on fibronectin (10 μg/mL) for 3 hours with or without 25 μmol TPCK. MCP-1 mRNA expression was assayed as described above. *$P < 0.05$; **$P < 0.001$ vs. suspension, respectively; †not significant vs. suspension. Abbreviations are: MCP-1, monocyte chemoattractant protein-1; MC, mesangial cells; TPCK, tosyl phenylalanyl chloromethylketone; PDTC, pyrrolidinedithiocarbamate; RT-PCR, reverse transcription polymerase chain reaction; HA, hemagglutinin; FAK, focal adhesion kinase.

tors; however, they exhibit different relative affinities depending on the substrate matrix. α1β1 integrin preferentially adheres to collagen type IV, whereas α2β1 integrin binds to collagen type I [44]. Both α1β1 and α2β1 bind to laminin as well as collagens, and α5β1 is a major fibronectin receptor [43]. Thus, although receptors for fibronectin, laminin, and collagens share structural components with β1 integrin, which directly binds to FAK, differences in the α subunit may account for the observed response. The differences in intracellular signaling molecules associated with different α subunit and/or expression levels of each α subunit might also be responsible for the observed differences between collagen types I, III, and IV. Because abnormal matrix remodeling characterized by increased expression of fibronectin, laminin, and collagen type IV, and de novo expression of collagen type I and III are the main pathologic features of progressive GN, it is interesting that collagen type IV, a normal component of glomeruli, had a lesser effect on MCP-1 induction compared to the pathologic collagens, types I and III.

MCP-1 is implicated in glomerular injury in various human and experimental GN, diabetic nephropathy, and hypertensive kidney disease [19, 20, 22, 45] by functioning as a major chemotactic factor for monocytes/macrophages [15]. Several signaling pathways are involved in the regulation of MCP-1 expression, such as MAP kinases, PKC, and NF-κB, which can also be activated by integrins [9, 37]. In the present study, adhesion-induced MCP-1 mRNA expression was completely inhibited by a tyrosine kinase inhibitor and TPCK, suggesting that tyrosine kinases, such as FAK and NF-κB are necessary in integrin-mediated induction of MCP-1. Furthermore, TPCK abolished wild-type FAK-induced enhancement of MCP-1 expression, suggesting that NF-κB is located downstream of FAK in integrin-mediated MCP-1 expression.

CONCLUSION

We have demonstrated in the present study that stimulation by ECM proteins per se, in addition to growth factors and cytokines, can induce MCP-1 expression in MC through the activation of the integrin/FAK cell adhesion signal pathway. Although the classic perception of MCP-1 is that its main function in glomerular diseases is initial attraction of monocytes/macrophages, overexpression of MCP-1 also induces certain intracellular signaling pathways that could lead to expression of various cytokines such as interleukin-6. Considered together, it is conceivable to view MCP-1 as an important mediator in the progression of renal diseases. We speculate, based on our data, that matrix accumulation might also play a role in maintaining monocyte infiltration after the development of ECM expansion in the subsequent phase of

glomerular diseases, although such accumulation might not be relevant to the initial monocyte infiltration into glomeruli. Collectively, expansion of ECM might be involved in the progression of glomerular diseases by manipulating the number of infiltrating monocytes and stimulation of cytokine gene expression through the induction of MCP-1 expression.

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